(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 229 045 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
07.08.2002 Bulletin 2002/32

(21) Application number: **01400255.4**

(22) Date of filing: **01.02.2001**

(51) Int Cl.[7]: **C07K 14/25**, C07K 19/00, C07K 17/00, C12N 15/31, C12N 1/21, C12Q 1/68, A61K 48/00, A61K 38/16

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(71) Applicants:
- **Institut Curie**
  75248 Paris Cedex 05 (FR)
- **Centre National De La Recherche Scientifique**
  75794 Paris Cedex 16 (FR)
- **INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)**
  75654 Paris Cédex 13 (FR)
- **UNIVERSITE PIERRE ET MARIE CURIE PARIS VI**
  75252 Paris Cédex 05 (FR)

(72) Inventors:
- **Johannes, Ludger**
  92400 Courbevoie (FR)
- **Tartour, Eric**
  75017 Paris (FR)
- **Goud, Bruno**
  75015 Paris (FR)
- **Fridman, Wolf Hervé**
  75005 Paris (FR)

(74) Representative: **Vaillant, Jeanne et al**
Ernest Gutmann - Yves Plasseraud SA,
3, rue Chauveau-Lagarde
75008 Paris (FR)

Remarks:
The sequence listing, which is published as annex to the application documents, was filed after the date of filing. The applicant has declared that it does not include matter which goes beyond the content of the application as filed.

(54) Universal carrier for targeting molecules to Gb3 receptor expressing cells

(57) The present invention concerns an universal polypeptidic carrier for targeting directly or indirectly a molecule to Gb3 receptor expressing cells and having the following formula STxB-Z(n)-Cys, wherein:

- STxB is the Shiga Toxin B subunit or a functional equivalent thereof,
- Z is an amino-acid devoided of sulfydryl group, n being 0, 1 or a polypeptide,
- Cys is the amino-acid Cysteine.

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Description

[0001] The invention relates to a universal polypeptidic carrier for targeting molecules to a Gb3 receptor for the B-subunit of Shiga-Toxin expressing cells and its use for intracellular transport and processing of said molecules.

[0002] Shiga Toxin is a bacterial toxin of the AB_5 subunit family that is secreted by *Shigella dysenteriae*. The A-subunit is the toxic moiety and inhibits the protein synthesis in higher eucaryotic target cells after transfering into the cytoplasm of said cells. The B-subunit is an homopentamer protein (5B - fragments) and is responsible for toxin binding to and internalization into target cells by interacting with the glycolipid Gb3 found on the plasma membranes of these cells. The B-fragment is non toxic, but conserves the intracellular transport characteristics of the holotoxin which, in many Gb3 expressing cells, is transported in a retrograde fashion from the plasma membranes to cytosol, via endosomes.

[0003] The glycolipid Gb3 receptor has also been reported to be expressed preferentially in some ectodermic derived tumors (plasma) and some Burkitt's lymphoma. It is also known as CD 77. In the present text, the term Gb3 should be considered as an equivalent to CD77.

[0004] The authors have already shown that a CD8 human tumor Antigen fused to the B subunit of Shiga toxin could efficiently be presented in an HLA class I-restricted manner to specific CTL (1). This result was independently confirmed by another study that demonstrated that Shiga holotoxin, carrying a defined peptide epitope from influenza virus, could deliver the antigen into the MHC class I intracellular pathway (3).

[0005] The authors have also shown that fusion proteins between the Gb3 receptor-binding non toxic B-fragment of bacterial Shiga toxin derived from Shigella dysenteriae and an antigen, or an epitope from a model tumor antigen, can elicit specific cytotoxic T lymphocytes response (CTL), whereas each moiety of said fusion protein does not lead individually to CTL induction (1, 2, and WO 99/03881).

[0006] The difficulty of this technology is that, for each application, i.e., for each antigen or fragment thereof, there is a need for a specific construction of a fusion protein, that necessitates a specific construction of a recombinant vector bearing the sequences encoding this fusion protein to be expressed in a host cell.

[0007] The aim of the present invention is to overcome the above-mentioned drawbacks and to provide a universal hook, or a universal carrier for targeting a molecule to a Gb3 receptor expressing cell to enable this molecule to be internalized, processed and/or expressed in said cell expressing Gb3 receptor.

[0008] In the present invention, a Shiga toxin B-subunit (STxB) derivative, or mutant, termed STxB-Cys has been designed. In this protein, a Cysteine is added at the C-terminus of mature STxB. The protein, when purified from bacteria, carries the internal disulfide bond, as wild type STxB, while the sulfhydryl group at the C-terminal Cys is free. Due to their nucleophilicity, free sulfhydryl groups are excellent acceptors for directed coupling approaches (4).

[0009] Thus, the present invention relates to a universal polypeptidic carrier for targeting directly or indirectly a molecule of interest to Gb3 receptor expressing cells having the following formula: STxB-Z(n)-Cys, wherein:

- STxB is the Shiga Toxin B subunit or a functional equivalent thereof,
- Z is an amino-acid devoided of sulfydryl group, n being 0, 1 or an amino-acid sequence,
- Cys being the amino-acid Cysteine.

[0010] The STxB moiety of the universal carrier has the sequence described in (8) or a functional equivalent thereof. A functional equivalent means a polypeptidic sequence having the capacity to bind specifically to the Gb3 receptor and/or to trigger an internalization of an antigen and its presentation in an MHC class-I restricted pathway.

[0011] In a preferred embodiment, n is 0 and the universal carrier has the following sequence (SEQ ID No 1):

COOH - MKKTLLIAASLSFFSASALATPDCVTGKVE

YTKYNDDDTFTVKVGDKELF

TNRWNLQSLLLSAQITGMTVTIKTNACHNGGGFSEVIFRC – NH2

[0012] As a matter of fact, if the Z linker is too long, i.e., when n is equal or greater than 2, some internal disulfide bridges might occur, and prevent either the binding of STxB to the Gb3 receptor and especially prevent the binding to the molecule of interest.

[0013] According to the invention, the molecule of interest is selected in the group constituted of proteins, peptides, oligopeptides, glycoproteins, glycopeptides, nucleic acids, polynucleotides, or a combination thereof.

[0014] In another aspect of the invention, the molecule of interest is an antigen to be targeted to antigen presentating

cells. Such cells are selected in a group comprising T lymphocytes, dendritic cells, macrophages Langerhans cells and the like.

[0015] In another aspect of the invention, the molecule of interest are drugs such as haptenes, psoralenes, or any compounds provided that they have a chemical group linkable with the -SH group of the Cysteine moiety of STxB-Cys.

[0016] The drug might be linked either directly or after activation with coumpounds such as bromoacetate, or any other method known by a skilled person, provided that the result of the reaction is a chemical entity having the following formula: STxB-Cys-M, M being all the above mentioned molecules of interest.

[0017] The coupling approaches for covalent binding of a peptidic or a polypeptidic moiety to STxB-Z(n)-Cys can be any method or processes described or carried out by a skilled person.

[0018] A first method that can be embodied is the use of SPDP hetero-bi-functional cross-linker described par Carlsson et al (5). However, SPDP is capable of being cleavable by serum thiolases that is a cause of decreasing the yield of the reaction. A second method for covalent coupling of STxB -Z(n)-Cys peptides with another peptide of interest is to produce bromoacetyl or maleimide functions on the latter as described by P. Schelte et al (4). Briefly, the peptide of interest is chemically activated with bromoacetate anhydride or by a maleimide group respectively. In appropriate reaction conditions (pH, temperature, incubation times), these groups are eliminated by cis-elimination, yielding respectively to -S-S, -S-CH_2-, or to -S-CO- or to -S-NH- covalents linkages.

[0019] As an example, the polypeptide or the peptide to be coupled to the -SH moiety the C-terminal Cysteine of the universal carrier, has its N-terminus activated with bromoacetic anhydride following the reaction scheme:

$$Br\text{-}CH_2\text{-}CO\text{-}O\text{-}CO\text{-}CH_2\text{-}Br + NH_2\text{-peptide} \Rightarrow Br\text{-}CH_2\text{-}CO\text{-}NH\text{-peptide} + Br\text{-}CH_2\text{-}COOH$$

[0020] The Bromoacetyl function has high chemoselectivity for peptide thiol groups and the activated peptide can be reacted with STxB-Cys as follows:

$$STxB\text{-}Cys\text{-}SH + Br\text{-}CH_2\text{-}CO\text{-}NH\text{-peptide} \Rightarrow STxB\text{-}Cys\text{-}S\text{-}CH_2\text{-}CO\text{-}NH\text{-peptide} + HBr$$

[0021] The resulting thioether-linkage is stable to hydrolysis.

[0022] Thus, another aspect of the invention is the product resulting from a covalent binding of STxB-Z(n)-Cys with a molecule of interest by a --S-S-, -S-CO-, or S-CH2- or -S-NH- linkage.

[0023] In one embodiment, the molecule of interest to be targeted to an antigen presentating cells is constituted by or comprises a polypeptidic structure, such an antigens or epitopes thereof, glycopeptides or glycoproteins, lipopeptides or lipoproteins.

[0024] In a preferred embodiment, the product resulting from the coupling of STxB-Z(n)-Cys with an antigen or a fragment thereof, where (n) is 0,1, or 2, and preferably 0, is able to be presented in an MHC class I restricted pathway.

[0025] In another embodiment, the molecule of interest is a polypeptide capable of binding with polynucleotide structures such as DNA or RNA molecules. Such molecules might be vectors or plasmids comprising a sequence of interest to be expressed in a target cell. In the present invention, a target cell is a eucaryotic cell bearing on its membrane the Gb3 receptor.

[0026] Thus, the universal carrier of the present invention is also a carrier for introducing a nucleotide sequence in a target cell either for gene therapy or for obtaining recombinant cells expressing heterologous proteins.

[0027] In another embodiment, the universal carrier according to the present invention can be operably linked directly through a covalent binding or indirectly through a linker to a cytotoxic drug to be targeted to tumor cells expressing Gb3 receptor.

[0028] The term "indirect binding" means that the universal carrier is covalently linked through the sulfhydryl moiety of the C-terminal Cysteine to a linker, said linker being operably linked to a drug or a pro-drug to be internalized into Gb3 receptor bearing cells.

[0029] This linkage might be a covalent binding or a non-covalent binding, provided that the affinity between the linker and the drug (or the pro-drug) is higher than 10^{-9}mole/l.

[0030] Another aspect of the invention is an isolated polynucleotide selected from the group of:

(a) a polynucleotide comprising the nucleotide sequence STxB encoding the Shiga Toxin B subunit or a functional equivalent thereof bearing at its 3'end the codon TGT, or the codon TGC encoding Cysteine;

b) a polynucleotide comprising a nucleotide sequence having at least 80% sequence identity to a nucleotide sequence encoding the Shiga Toxin B subunit or a functional equivalent thereof bearing at its 3'end the codon TGT or TGC; and

c) a nucleotide sequence complementary to the sequence in a) or b).

[0031] In a preferred embodiment, the polynucleotide has the following SEQ ID N° 2:

5' - atgaaaaaaacattattaatagctgcatcgctttcatttttttcagcaag

tgcgctggcgacgcctgattgtgtaactggaaaggtggagtatacaaaat

ataatgatgacgatacctttacagttaaagtgggtgataaagaattattt

accaacagatggaatcttcagtctcttcttctcagtgcgcaaattacggg

gatgactgtaaccattaaaactaatgcctgtcataatggaggggggattca

gcgaagttatttttcgttgt – 3'

[0032] The present invention relates also to a recombinant vector or to a plasmid comprising a polynucleotide sequence as described above, and capable of expressing the universal carrier STxB-Z(n)-Cys, where (n) is 0,1 or 2, STxB and Z have the same significance as above, in an appropriate host cell.

[0033] As an example, a convenient vector is the plasmid pSu108 described in (7).

[0034] Another object of the present invention is to provide a method for obtaining a plasmid expressing STxB-Z(n) -Cys comprising:

a) providing a plasmid comprising a STxB sequence;
b) applying two PCR amplification steps using two couples of primers, AA' and BB',

- A and B being complementary to each other and comprising the Cys codon,
- A' and B' being outside the STxB sequence;

c) isolating the amplified fragments;
d) hybridizing the amplified fragments;
e) applying a PCR amplification on the hybridized fragments;
f) insertion of the amplified fragment into a plasmid.

[0035] In a preferred embodiment, the plasmid pSU108 (7) containing STxB fragment was modified to introduce the Cysteine codon TGT at the 3' end of the B-fragment cDNA. The primers for step b) are respectively for AA' and BB':

- primer A: 5'-AGCGAAGTTATTTTTCGTTGTTGACTCAGAATAGCTC-3' (SEQ ID 3), and
- primer B: 5'-GAGCTATTCTGAGTCAACACGAAAAATAACTTC-3' (SEQ ID n° 4).
- primer A': primer ShigaAtpE: 5'-CACTACTACGTTTTAAC-3' (SEQ ID n° 5), and
- primer B': primer Shiga-fd: 5'-CGGCGCAACTATCGG-3' (SEQ ID n° 6).

[0036] The PCR of step e) yields a fragment that is cloned into the SphI and SaI1 restriction sites of pSU108. Sequences derived by PCR are verified by dideoxy-sequencing.

[0037] The skilled person can easily design the choice of primers, plasmids for producing a vector bearing the polynucleotide sequence expressing STxB-Z(n)-Cys in an appropriate host cell, provided that this succession of steps allows the interpretation of the Cys codon into the amplified fragment.

[0038] The invention also provide a recombinant cell line obtained by transformation with the recombinant vector containing the polypeptide sequence encoding the universal carrier as described above. In a preferred embodiment, said recombinant cell line is a procaryotic cell, preferentially E. coli.

[0039] In a still preferred embodiment, the plasmid is pSU108 having SEQ ID No. 2 integrated between the SphI and SalI restriction sites, and the corresponding cell line has been deposited at CNCM on December 19, 2000 with the registration number 1-2604.

[0040] The present invention also provide a process for producing a universal carrier as described above comprising:

a) culturing a recombinant cell line as described above,

b) obtaining a periplasmic extract of said cells, and
c) purifying said polypeptide.

[0041] Preferentially, the cell line is E. coli and in c) the purification is made by anion exchange column chromatography followed by a gel filtration column chromatography.

[0042] Such a process is particularly advantageous for large scale production of the universal carrier, as far as it can then be operably linked by covalent coupling with a molecule of interest, and used within a large scope of application.

[0043] The present invention also provides a method for delivering a sequence of interest into the MHC class I pathway using a product obtained by covalent binding of the Cys moiety of the universal carrier with said sequence of interest; this method is advantageous to elicit a CTL response to a given antigen or epitope thereof as far as the product is specific to the cell involved in the MHC class I pathway.

[0044] As a matter of fact, the inventors have shown that an immunodominant peptide, derived from the ovalbumin protein, and coupled chemically to STxB-Cys, could be presented by antigen presenting cells to specific hybridoma cells, demonstrating that STxB could deliver exogenous immunogenic peptide in the MHC class I pathway. To exclude a bias due to the presence of free peptides contaminating the material, experiments using fixed dendritic cells clearly demonstrated that the internalization of the fusion protein was required for this process. The inventors also have shown that the Shiga toxin receptor, Gb_3, was also involved in the ability of STxB-Cys to target exogenous peptide in the endogenous MHC class I pathway.

[0045] The invention also pertains to a method for delivering an expression vector containing a sequence of interest into a Gb3 receptor expressing cells characterized in that said expression vector is bound to a lysine rich peptide covalently linked to the Cys moiety of the universal carrier.

[0046] As an example, the lysine rich peptide is a 16-mer poly-lysine which is able to bind any polynucleotidic sequence, either of DNA or RNA nature. Such a peptide carrying a 16-mer of lysines will be activated at its N-terminus by bromoacetate anhydride and coupled to STxB-Cys. Expression plasmids will be bound to this coupling product, and vectorization of DNA into target cells is assayed using convenient reporter systems, such as the green fluorescent protein or luciferase.

[0047] The capacity to target expression plasmids with the help of STxB to the nucleus of antigen presenting cells is expected to further improve the power of this vector, since i) DNA can even more easily be adopted to new experimental or clinical needs, and ii) due to its potentiation effect, expression of antigenic peptides or proteins from DNA would further increase the sensitivity of STxB-dependent antigen presentation.

[0048] The invention also provides a method for delivering a drug or a pro-drug into a cell, particularly into a cancer cell bearing Gb3 (or CD77) receptor.

[0049] The glycolipid Gb_3 receptor has been reported to be preferentially expressed in some neuroectodermic derived tumors (glioma) and some Burkitt's lymphoma. Since one limitation of the use of chemotherapy in cancer is secondary side effects of the drugs because of their toxicity on normal cells, the drugs are preferentially vectorized in tumor cells by using STxB-Cys. The drugs are activated to become reactive with the sulfhydryl group of STxB-Cys. To achieve this, a maleimide group can be introduced on a drug, for example psoralenes compounds.

[0050] The present invention also pertains to:

- a pharmaceutical composition for enhancing the immunogenicity of a peptide or a protein or a glycoprotein or a lipopeptide, containing the universal carrier covalently linked by its Cys moiety to said peptide or protein or glycoprotein or lipopeptide;
- a pharmaceutical composition for treating tumor cells bearing the Gb3 receptor (CD77), containing the universal carrier according to the invention covalently linked by its Cys moiety to a drug or a pro-drug toxic for said tumor cells.

[0051] Without limiting the scope of the universal carrier of the invention and its widespread use in different applications, the hereinafter examples and figures illustrate the advantages of the present invention.

Legend of the figures:

[0052] Figure 1 represents the protein profile of the final SephaDex 75 column yielding purified STxB-Cys. Fractions 20-25 contain mostly monomeric STxB-Cys (the positions of monomeric and dimeric STxB-Cys are indicated to the right). Molecular weight markers are indicated to the left.

[0053] Figure 2a represents the coupling of Type2 of Pep2 [as defined in example 2] to STxB-Cys, followed by an in vitro antigen presentation assay on D1 dendritic cells, as described in (2). Two different preparations of STxB-Cys coupled to the SL8 peptide, an immunodominant epitope of ovalbumin were used (termed 4A and 9A).

[0054] Figure 2b represents a control experiment of figure 2a, in which it is shown that fixed D1 can still present free SL8 peptide.

[0055] Figure 3 represents another experiment on fixed and non fixed D1 cells using a coupling reaction of Type1 on STxB-SH and Pep1 [as defined in example 2].

[0056] Figure 4 represents the B-subunit dependent presentation of antigenic peptides derived from a coupling of Pep2 to B-Glyc-Cys-KDEL.

[0057] Figure 5 shows that the Gb3 synthesis inhibitor PPMP inhibits B-subunit dependent antigen presentation (5a) and that SL8 presentation is not decreased in PPMP treated cells.

Example 1: Preparation of the universal carrier

a) Construction of a plasmid expressing STxB-Cys:

[0058] In a preferred embodiment, the plasmid pSU108 (7) was modified to introduce the Cysteine codon tgt at the 3' end of the B-fragment cDNA. PCR primer A: SEQ ID n° 3 (5'-AGCGAAGTTATTTTTCGTTGTTGACTCAGAAT-AGCTC-3') and primer A': SEQ ID n° 4 (5'-GAGCTATTCTGAGTCAACACGAAAAATAACTTC-3') were used with plasmid specific primers ShigaAtpE: SEQ ID n° 5 (5'-CACTACTACGTTTTAAC-3') and Shiga-fd: SEQ ID n° 6 (5'-CGGCG-CAACTATCGG-3') to produce DNA fragments which, in a second PCR with primers Shiga AtpE and Shiga-fd yielded a fragment that was cloned into the SphI and Sal1 restriction sites of pSU108. Sequences derived by PCR were verified by dideoxy-sequencing.

b) Protein purification:

[0059]

b) 1. Preparation of the periplasmic extract was performed as follows:

- Inoculate 125 ml of LB/Amp with 125µl of an overnight culture grown at 30°C,
- grow over night at 30°C,
- transfer into 375 ml of LB/Amp at 50°C; incubate 4 hours at 42°C,
- centrifuge to pellet cells,
- wash cells 3 times with 10 mM Tris/HC1, pH 8.0,
- re-suspend cells in 200 ml of 25% sucrose, 1 mM EDTA, 10mM Tris/HC1, Ph 8.0; incubate at room temperature for 10 min.,
- centrifuge to pellet cells,
- re-suspend cells in 200 ml of ice cold water containing a protease inhibitor cocktail; incubate on ice for 10 min.,
- centrifuge; collect supernatant; add 20 mM Tris/HC1, Ph8.0.

b) 2. Purification on columns:

The periplasmic extract was loaded on a QFF anion exchanger column (pharmacia) and eluted at 230 mM Nacl. STxB-Cys containing fractions are pooled, diluted 4-fold and loaded on a Mono Q anion exchanger column (pharmacia), followed by elution at 230 mM Nacl. After concentration with microconcentration devices from Pall-Filtron, the pooled fractions were passed through a Sephadex 75 gel filtration column. Purity was above 95% (Fig. 1).

b) 3. Product characterization:

The B-fragments of STxB-Cys, purified from Sephadex 75 gel filtration columns, are essentially monomeric (Fig. 1). This is in marked difference to constructions where the Cysteine was added at more than 2 amino acids from the natural C-terminus of the B-fragment. In these cases, neighbouring B-fragments within a pentamer are engaged in disulfide bonds.

Example 2: Conditions for coupling of activated peptides to the universal carrier:

a) Carriers:

[0060] Three different carriers have been compared.

1) STxB-Cys: B-fragment to which a Cys has been added right to its C-terminus. This protein elutes as a monomer from the purification columns.

2) STxB-Z_2-Cys: carrier with a short spacer (2 amino acids resulting from a cloning cassette) between the C-terminus of the wild type B-fragment and the Cys. The majority of the protein eluted as dimers from the purification

columns. These can be separated under reducing conditions, indicating the formation of disulfide bonds between monomers in the pentameric B-subunit complex.
3) STxB-Glyc-Cys-KDEL: carrier in which the Cys is located between a Glycosylation cassette being 9 amino acid long and a C-terminal KDEL peptide. The majority of the protein eluted as dimers from the purification columns. These can be separated under reducing conditions, indicating the formation of disulfide bonds between monomers in the pentameric B-subunit complex.

b) test peptides:

[0061]

1) Pep1: a synthetic peptide of 16 amino acids carrying the SL8 antigenic peptide derived from chicken ovalbumin.
2) Pep2: a synthetic peptide of 24 amino acids as above with, in addition, a His-gag at its C-terminus.
3) SL8: the antigenic peptide from ovalbumin that can directly exchange with peptides on MHC class I complexes at the plasma membrane of antigen presenting cells.

c) Coupling conditions:

[0062] Under reducing conditions (Type1): Fusion proteins were treated with DTT overnight, then activated peptide (carrying a bromo acetate group at its N-terminus) was added in excess. Conditions used for the first coupling experiments using fusion proteins will mostly dimerize monomers (proteins STxB-Z_2-Cys and STxB-Glyc-Cys-KDEL).
[0063] Under non-reducing conditions (Type2): Fusion proteins are directly reacted with the activated peptides.

d) Biochemical and morphological controls:

[0064] Pep2 carries a His-tag. This has allowed us, using an anti-His antibody, to show the presence of Pep2 on B-subunit by Western blotting, and the B-subunit dependent transport of Pep2 in HeLa cells.
[0065] Figure 2a shows that a dose dependent stimulation of the B3Z CTL hybridoma (measurement of β-galactosidase activity) was observed with non-fixed cells, while fixation abolished antigen presentation.
[0066] Note that antigen presentation only works on non-fixed cells, indicating that the observed presentation does not result from contaminating free Pep2.
[0067] Figure 2b shows the control experiment of figure 2a in which it is shown that fixed D1 cells can still present free SL8 peptide.
[0068] In figure 3, it appears that in this type of protocol, some free Pep1 appears to co-purify with the fusion protein, since at high doses (200-1000nM), some presentation was observed on fixed cells. Presentation by SL8 is shown to the right.
[0069] In figure 4, the coupled protein (lanes 1 and 2) or the SL8 Peptide (lanes 5 and 6) were incubated (lanes 2 and 5) or not (lanes 1 and 4) with anti-B-subunit Fab-fragment derived from the 13C4 antibody which inhibits the binding of the B-subunit to Gb3. Note that the Fab-fragment neutralizes the capacity of the B-subunit to introduce the antigenic peptide into the class I pathway, while the presentation with SL8 is not affected under these conditions. The background signal in this experiment was at 0.3.
[0070] In figure 5a, D_1 cells were pre-treated with PPMP (see fig. 3b of (2)) for 3 days. This treatment lead to an important decrease of Gb3 expression at the cell surface, without however eliminating it completely. Under this condition, antigen presentation from a coupling reaction of Pep1 with STxB-Glyc-Cys-KDEL was significantly reduced, indicating that Gb3 is important for the presentation phenomena.
[0071] It appears from all these experiments that the coupling under non-reducing to STxB-Cys is surprisingly efficient (in terms of sensitivity; note that, as shown in fig. 1, only 4nM of STxB-Cys-Pep2 are necessary to have a response). Thus, the universal carrier STxB-Cys is preferred due to its simplicity in its preparation and to the reproducibility of the coupling.
[0072] Hence, the optimal conditions for coupling of activated peptides to STxB-Cys were the following:

- dialyse STxB-Cys against 20mM Borate buffer, pH 9.0, 150mM NaCl,
- concentrate to 1 mg/ml,
- dissolve N-terminally activated peptide (activated with bromoacetate anhydride) at 12mM in DMSO,
- dilute peptide to 0.2mM in protein solution,
- incubate 12 hours at room temperature,
- dialyse against PBS.

Example 3: Characterization of STxB as to its antigen presentation capacity

[0073] The following experimental series will help to fully describe the capacity of STxB to function in antigen presentation system.

a) Class I- and class II-restricted antigen presentation:

[0074] A peptide carrying class I- and II- restricted antigenic peptides from chicken ovalbumin (Br-CH2-CO-NH-LEQLESIINFEKLTEWSLKISQAVHAAHAEINEAGR, sequences 257-264 and 323-339 were coupled to STxB-Cys, and the class I- and class II-restricted presentation of these peptides were assayed using the corresponding T-cell hybridomas.

b) Size evaluation of sequences vectorized by STxB.

Peptides of increasing length were purchased carrying the repetitive sequence element NH2-QLESIINFKLTEW-COOH, a class I restricted epitope from ovalbumin (amino acids 254-267). These were coupled to STxB-Cys, and were tested whether antigen presentation efficiency evolves in the function of peptide length.

c) Coupling of whole size proteins.

Our preliminary evidence suggests that chicken ovalbumin can be coupled to STxB-Cys. These experiments have been done with the SPDP heterobifunctional cross-linker. (Carlsson et al., 1978).

A first series of antigen presentation experiments indicated that the ovalbumin protein can be introduced into the endogenous MHC class I-restricted antigen presentation pathway of mouse dendritic cells. SPDP has the inconvenience of being cleavable by serum thiolases. This cross-linker was successfully substituted by MBS which is non-cleavable. Other antigenic proteins (Mart 1 and polypeptides derived from HPV16-E7 and Muc1) were tested to show that the procedure is of universal use.

d) Coupling of complex protein mixtures.

[0075] A lysate from the cervix carcinoma-derived cell line Caski is used. This cervix carcinoma cell line, which expresses the HLA-A2 allele at its membrane, also expresses Human papillomavirus derived peptides. E7 is a early transcribed ORF from HPV which is necessary for transformation of primary keratinocytes. Since anti-E7 HLA A2-restricted CTL are elicited *in vitro*. The efficacy of the coupling of this protein mixture by a presentation assay specific for HLA-A2 E7 derived peptides was tested. As control, a lysate from a HLA-A2-positive cell line which does not express E7 (croft cells or Daudi) was coupled to STxB-lys.

Example 4: Application to MHC class I-restricted antigen presentation

[0076] The experiment of figure 4 shows that STxB-Cys dependent antigen presentation is inhibited when the interaction with Gb_3 is abolished. Here it is found that pre-binding of the Fab fragment of monoclonal Ab against STxB to 0.1 μM STxB-Cys, coupled to SL8, inhibited antigen presentation, suggesting that STxB-Cys binding to Gb_3 is necessary for antigen presentation. Similar results were obtained when Gb_3-expression was inhibited with a drug (not shown).

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(7) Johannes, L., Tenza, D., Antony, C. and Goud, B., "Retrograde transport of KDEL-bearing B-fragment of Shiga toxin". (1997) J. Biol. Chem. 272: 19554-19561.
(8) N. A. Stockbine, M. P. Jackson, L.M. Sung, R.K. Holmes, A.D. O'Brien, *J* Bacteriol 170, 1116-22 (1988).

Annex to the application documents - subsequently filed sequences listing

[0078]

SEQUENCE LISTING

<110> INSTITUT CURIE
CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE
INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE M
UNIVERSITE PIERRE ET MARIE CURIE (PARIS VI)

<120> UNIVERSAL CARRIER FOR TARGETING MOLECULES TO Gb3 RECEPTOR EXPRESSING CELLS

<130> B4719 - FL/SDU (Institut Curie et al)

<140> 01400255.4
<141> 2001-02-01

<160> 6

<170> PatentIn Ver. 2.1

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<211> 90
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Universal carrier

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Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr
             20                  25                  30

Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu
         35                  40                  45

Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln
     50                  55                  60

Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
 65                  70                  75                  80

Gly Gly Phe Ser Glu Val Ile Phe Arg Cys
                 85                  90
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acagttaaag tgggtgataa agaattattt accaacagat ggaatcttca gtctcttctt 180
ctcagtgcgc aaattacggg gatgactgta accattaaaa ctaatgcctg tcataatgga 240
gggggattca gcgaagttat ttttcgttgt 270

<210> 3
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<212> DNA
<213> Artificial Sequence

<220>
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<210> 4
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<220>
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<220>
<223> Description of Artificial Sequence: Primer A'

<220>
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<210> 6
<211> 15
<212> DNA
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<220>
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<220>
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<222> (1)..(15)
<223> Primer Shiga-fd

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cggcgcaact atcgg 15

Claims

1. Universal polypeptidic carrier for targeting directly or indirectly a molecule to Gb3 receptor expressing cells and having the following formula STxB-Z(n)-Cys, wherein:

   - STxB is the Shiga Toxin B subunit or a functional equivalent thereof,
   - Z is an amino-acid devoided of sulfydryl group, n being 0, 1 or a polypeptide,
   - Cys is the amino-acid Cysteine.

2. Universal carrier according to claim 1 wherein n is 0.

3. Universal carrier according to claim 1 or 2 wherein the molecule is selected in the group constituted of proteins, peptides, oligopeptides, glycoproteins, glycopeptides, nucleic acids, polynucleotides, or a combination thereof.

4. Universal carrier according to claim 1 or 2 wherein the molecule is covalently linked to the -S residue of the universal carrier by a -S-S-, or -S-CO-, or -S-CH2-. Or -S-NH- linkage.

5. Universal carrier according to claim 4 wherein the molecule is an antigen to be targeted to antigen presentating cells.

6. Universal carrier according to claim 1 or 2 wherein the universal carrier is covalently linked to an oligopeptide or a polypeptide by a -S-S-, or -S-CO-, or -S-CH2- or -S-NH- linkage, and the molecule to be targeted is operably linked to the said oligopeptide or polypeptide.

7. Universal carrier according to claim 6 **characterized in that** it is covalently linked to a poly-lysine oligopeptide and the molecule to be targeted is a nucleic acid or an oligonucleotide operably linked to the said poly-lysine moiety.

8. Universal carrier according to claim 4 wherein the molecule is a cytotoxic drug or pro-drug to be targeted to tumor cells expressing Gb3 receptor.

9. An isolated polynucleotide selected from the group consisting of:

   a) a polynucleotide comprising the nucleotide sequence STxB encoding the Shiga Toxin B subunit or a functional equivalent thereof bearing at its 3'end the codon TGT, or the codon TGC encoding Cysteine,
   b) a polynucleotide comprising a nucleotide sequence having at least 80% sequence identity to a nucleotide sequence encoding the Shiga Toxin B subunit or a functional equivalent thereof bearing at its 3'end the codon TGT or TGC,
   c) a nucleotide sequence complementary to the sequence in a) or b).

10. A polynucleotide according to claim 9 having the SEQ ID NO 2.

11. A recombinant vector, or plasmid, comprising a polynucleotide sequence according to claim 9 or 10, for the expression of the universal vector of claim 1 in an appropriate host cell.

12. A recombinant cell line obtained by transformation with the recombinant vector according to claim 11.

13. A recombinant cell line according to claim 12 being a procarytic cell line, preferably E. coli.

14. A recombinant cell line according to claim 11, 12 or 13, deposited at CNCM on December 19, 2000, with the registration number I-2604.

15. A method for constructing a recombinant vector according to claim 11 or 12 comprising:

   a) providing a plasmid comprising STxB sequence;
   b) applying two PCR amplification steps using two couples of primers, AA' and BB',

   - A and B being complementary to each other and comprising the Cys codon,
   - A' and B' being outside the STxB sequence;

c) isolating the amplified fragments;
d) hybridizing the amplified fragments;
e) applying a PCR amplification on the hybridized fragments;
f) insertion of the amplified fragment into a plasmid.

16. A method according to claim 15 wherein in step f) the fragment is inserted into the Sph1 and Sal1 restriction site of the plasmid pSU108.

17. A process for producing a purified polypeptide according to claim 1 comprising:

a) culturing the cell line according to anyone of claim 12 to 14,
b) obtaining a periplasmic extract of said cells
c) purifying said polypeptide.

18. A process according to claim 17 wherein in a), the cell line is E.coli and in c) the purification is made by anion exchange column chromatography followed by a gel filtration column chromatography.

19. Method for delivering an sequence of interest into the MHC class I pathway using the a product obtained by covalent binding of the Cys moiety of the universal carrier with said sequence of interest.

20. Method for delivering an expression vector containing a sequence of interest into a Gb3 receptor expressing cells **characterized in that** said expression vector is operably linked to a lysine-rich peptide covalently linked to the Cys moiety of the universal carrier.

21. Method according to claim 20 wherein the lysine rich peptide is a 16-mer poly-lysine.

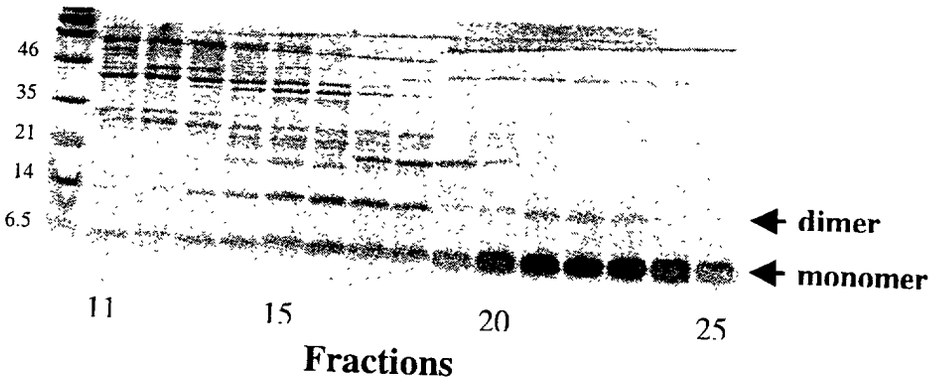
22. Method according to claim 19 to 21 wherein the sequence of interest is selected amongst:

- a sequence encoding an immunogenic peptide, or
- a sequence encoding a drug or a pro-drug becoming toxic for the Gb3 receptor expressing cells, or
- a sequence encoding a therapeutic active molecule.

23. Pharmaceutical composition for enhancing the immunogenicity of a peptide or a protein or a glycoprotein or a lipopeptide, containing the polypepidic carrier according to claim 1 or 2 covalently linked by its Cys moiety to said peptide or protein or glycoprotein or lipopeptide.

24. Pharmaceutical composition for treating tumor cells bearing the Gb3 , containing the polypeptidic carrier according to claim 1 or 2 covalently linked by its Cys moiety to a drug or a prodrug toxic for said tumor cells.

Fig. 1


46
35
21
14
6.5
dimer
monomer
11
15
20
25
Fractions

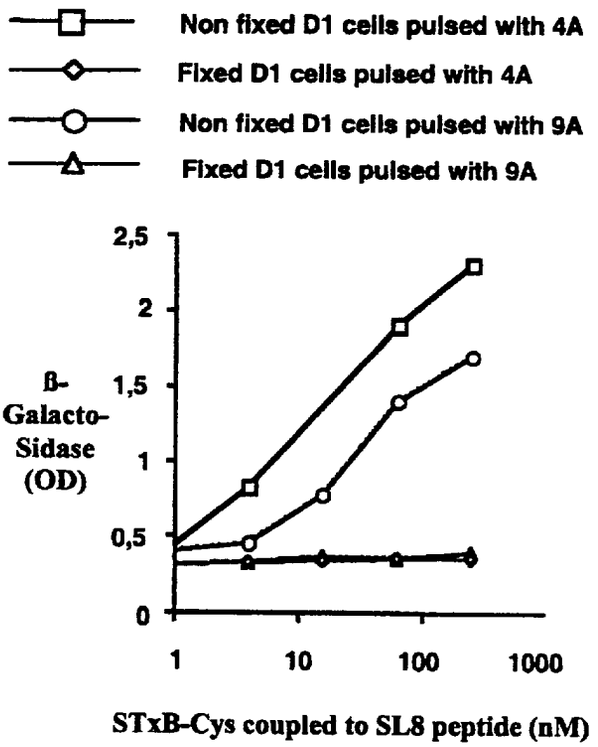
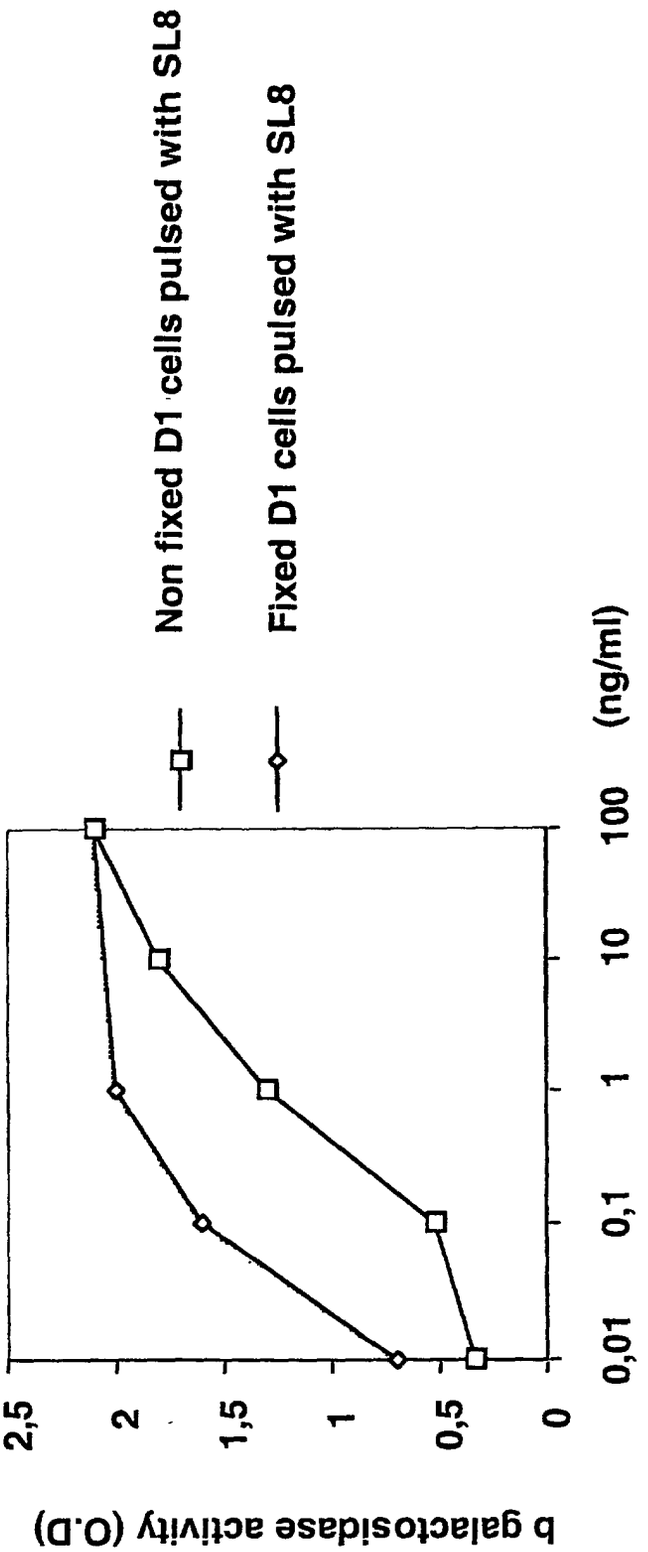

Non fixed D1 cells pulsed with 4A
Fixed D1 cells pulsed with 4A
Non fixed D1 cells pulsed with 9A
Fixed D1 cells pulsed with 9A
ß-Galacto-Sidase (OD)
2,5
2
1,5
1
0,5
0
1
10
100
1000
STxB-Cys coupled to SL8 peptide (nM)

Fig. 2a

Fig. 2b


Non fixed D1 cells pulsed with SL8
Fixed D1 cells pulsed with SL8
b galactosidase activity (O.D)
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2
1,5
1
0,5
0
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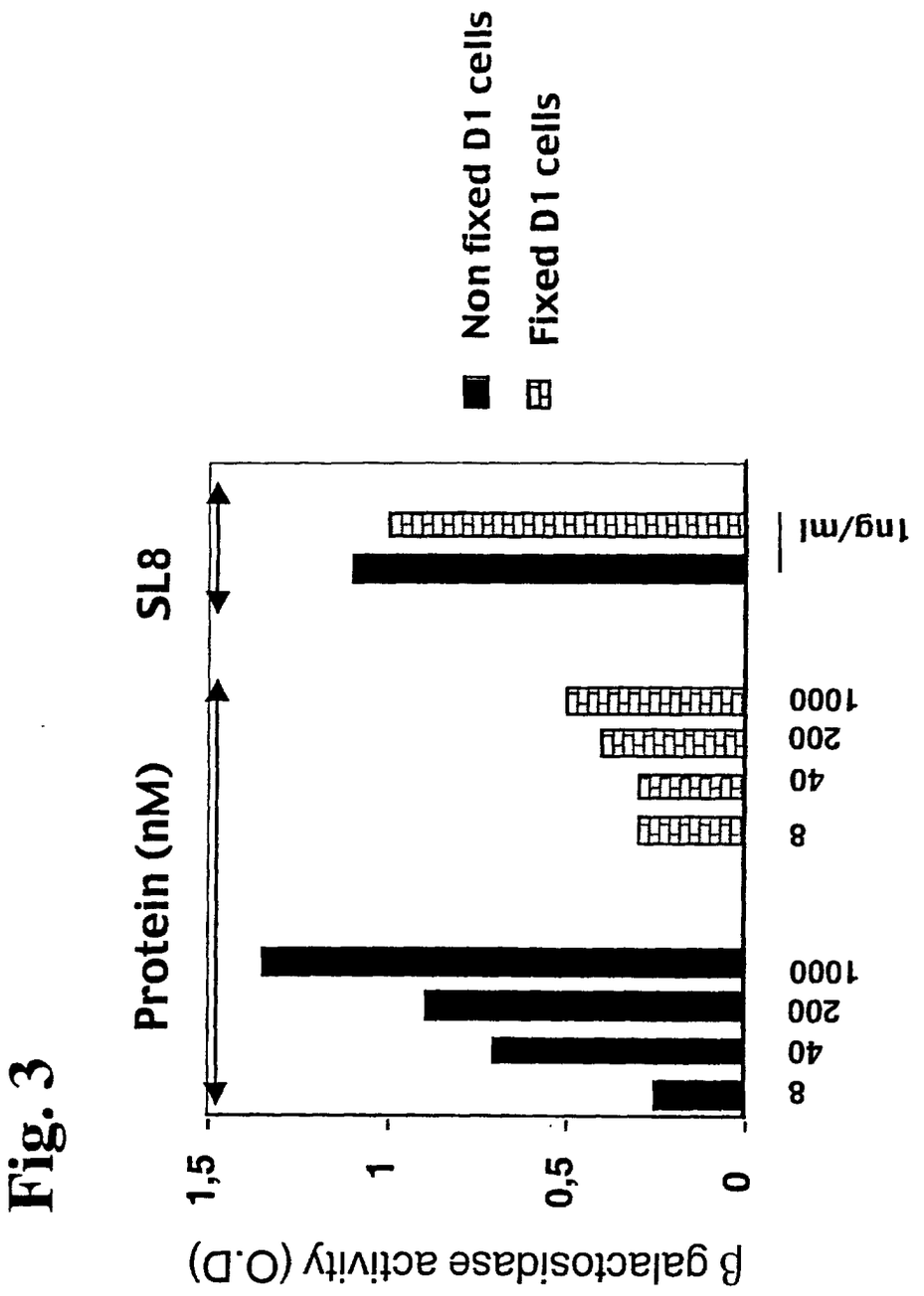
0,1
1
10
100
(ng/ml)

Fig. 3


Non fixed D1 cells
Fixed D1 cells
β galactosidase activity (O.D)
1,5
1
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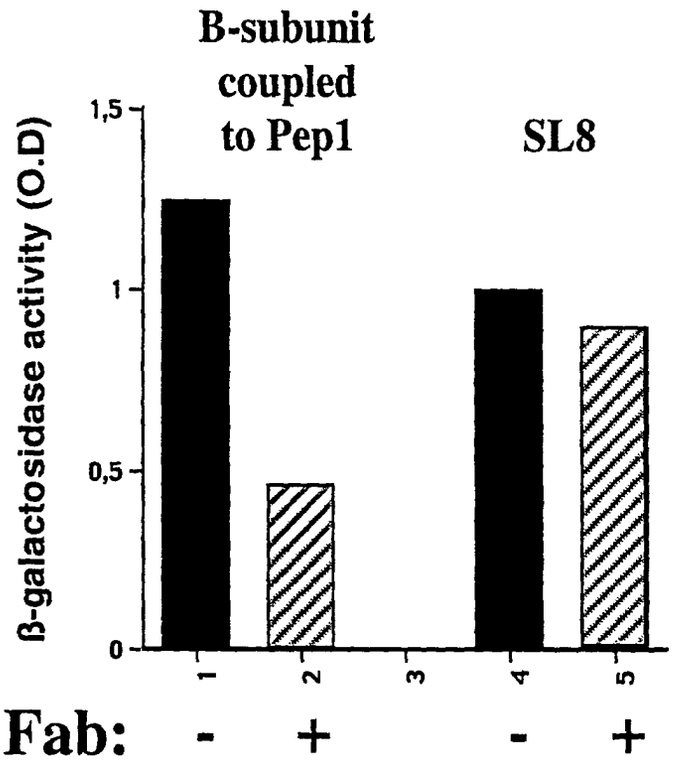
0
Protein (nM)
SL8
8
40
200
1000
8
40
200
1000
1ng/ml

Fig. 4


B-subunit
coupled
to Pep1
SL8
ß-galactosidase activity (O.D)
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1
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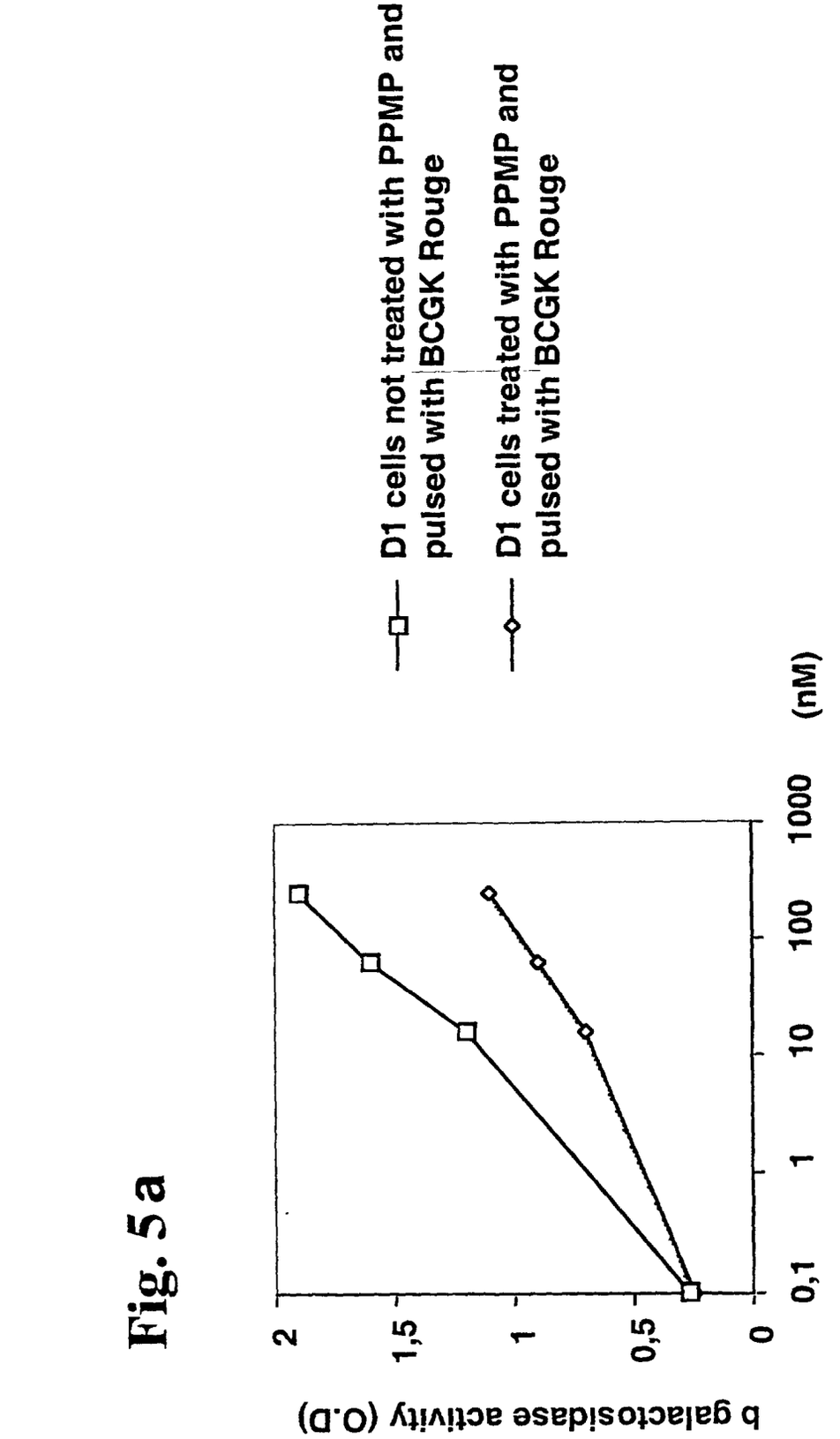
1
2
3
4
5
Fab:
-
+
-
+

Fig. 5a


D1 cells not treated with PPMP and pulsed with BCGK Rouge
D1 cells treated with PPMP and pulsed with BCGK Rouge
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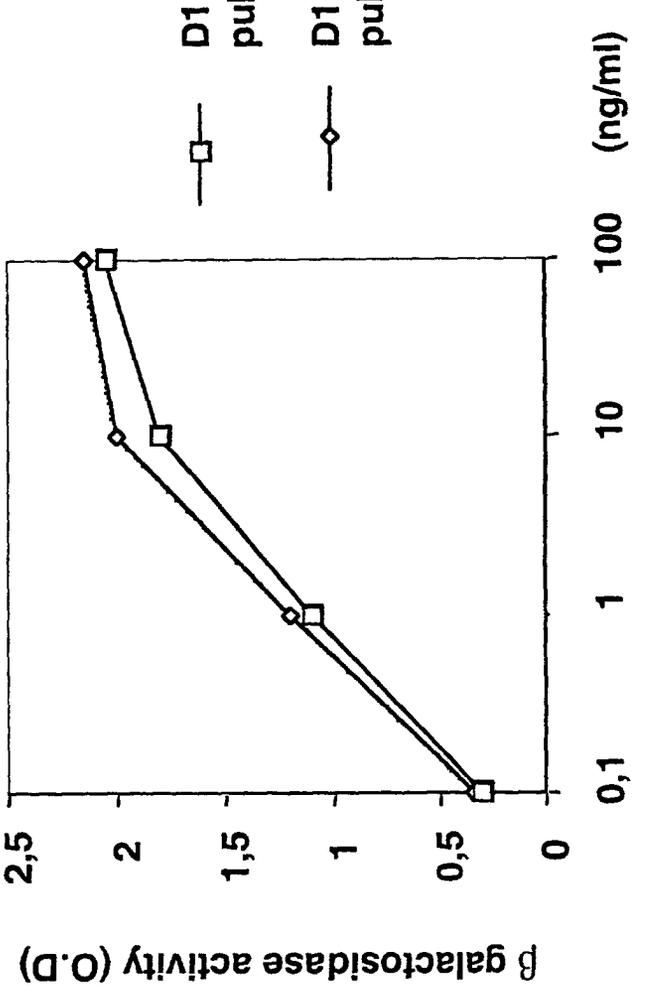
0
0,1
1
10
100
1000
(nM)

Fig. 5b


β galactosidase activity (O.D)
2,5
2
1,5
1
0,5
0
0,1
1
10
100
(ng/ml)
D1 cells treated with PPMP and pulsed with SL8
D1 cells not trated with PPMP and pulsed with SL8

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 01 40 0255

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
D,A	WO 99 03881 A (INSTITUT CURIE) 28 January 1999 (1999-01-28) * page 3, line 1 - page 5, line 11 * * page 5, line 26 - page 9, line 29 * ---	1-24	C07K14/25 C07K19/00 C07K17/00 C12N15/31 C12N1/21
D,A	LEE R-S ET AL: "MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I PRESENTATION OF EXOGENOUS SOLUBLE TUMOR ANTIGEN FUSED TO THE B-FRAGMENT OF SHIGA TOXIN" EUROPEAN JOURNAL OF IMMUNOLOGY, WEINHEIM, DE, vol. 28, no. 9, September 1998 (1998-09), pages 2726-2737, XP000856227 ISSN: 0014-2980 * abstract * * page 2727, right-hand column, last paragraph - page 2729, right-hand column, paragraph 1 * --- -/--	1-24	C12Q1/68 A61K48/00 A61K38/16

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

C07K
C12N
C12Q
A61K

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

Although claims 19-22, as far as concerning an in-vivo method, are directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

Place of search	Date of completion of the search	Examiner
THE HAGUE	18 January 2002	Montero Lopez, B

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 01 40 0255

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
D,A	NOAKES KL ET AL: "EXPLOITING RETROGRADE TRANSPORT OF SHIGA-LIKE TOXIN 1 FOR THE DELIVERY OF EXOGENOUS ANTIGENS INTO THE MHC CLASS I PRESENTATION PATHWAY" FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 453, no. 1/2, 18 June 1999 (1999-06-18), pages 95-99, XP001015751 ISSN: 0014-5793 * the whole document * ---	1-24	
D,A	NACILLA HAICHEUR ET AL.: "The B subunit of Shiga toxin fused to a tumor antigen elicits CTL and targets dendritic cells to allow MHC class I-restricted presentation of peptides derived from exogenous antigens" JOURNAL OF IMMUNOLOGY, vol. 165, September 2000 (2000-09), pages 3301-3308, XP002187748 * abstract * * page 3302, right-hand column, paragraph 5 - page 3303, left-hand column, paragraph 7 * ---	1-24	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
	-/--		

EPO FORM 1503 03.82 (P04C10)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 01 40 0255

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	SCHELTE P ET AL: "TECHNICAL NOTES. DIFFERENTIAL REACTIVITY OF MALEIMIDE AND BROMOACETYL FUNCTIONS WITH THIOLS: APPLICATION TO THE PREPARATION OF LIPOSOMAL DIEPITOPE CONSTRUCTS" BIOCONJUGATE CHEMISTRY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, US, vol. 11, no. 1, January 2000 (2000-01), pages 118-123, XP000887774 ISSN: 1043-1802 * page 118, left-hand column, paragraph 1 - page 119, left-hand column, paragraph 2 *	1-24	
A	SHIGEMATSU H ET AL: "Site-directed lipid modification of IgG-binding protein by intracellular bacterial lipoprotein process" JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 75, no. 1, 24 September 1999 (1999-09-24), pages 23-31, XP004202724 ISSN: 0168-1656 * abstract *	1-24	TECHNICAL FIELDS SEARCHED (Int.Cl.7)

EPO FORM 1503 03.82 (P04C10)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 01 40 0255

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

18-01-2002

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9903881 A	28-01-1999	FR 2766193 A1	22-01-1999
		AU 8812498 A	10-02-1999
		CN 1272882 T	08-11-2000
		EP 1017715 A2	12-07-2000
		WO 9903881 A2	28-01-1999
		JP 2001510030 T	31-07-2001

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82